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Saturated Fatty Acid Activates but Polyunsaturated Fatty Acid Inhibits Toll-like Receptor 2 Dimerized with Toll-like Receptor 6 or 1*

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Toll-like receptor 4 (TLR4) and TLR2 agonists from bacterial origin require acylated saturated fatty acids in their molecules. Previously, we reported that TLR4 activation is reciprocally modulated by saturated and polyunsaturated fatty acids in macrophages. However, it is not known whether fatty acids can modulate the activation of TLR2 or other TLRs for which respective ligands do not require acylated fatty acids. A saturated fatty acid, lauric acid, induced NFκB activation when TLR2 was co-transfected with TLR1 or TLR6 in 293T cells, but not when TLR1, 2, 3, 5, 6, or 9 was transfected individually. An *n*-3 polyunsaturated fatty acid (docosahexaenoic acid (DHA)) suppressed NFκB activation and cyclooxygenase-2 expression induced by the agonist for TLR2, 3, 4, 5, or 9 in a macrophage cell line (RAW264.7). Because dimerization is considered one of the potential mechanisms for the activation of TLR2 and TLR4, we determined whether the fatty acids modulate the dimerization. However, neither lauric acid nor DHA affected the heterodimerization of TLR2 with TLR6 as well as the homodimerization of TLR4 as determined by co-immunoprecipitation assays in 293T cells in which these TLRs were transiently overexpressed. Together, these results demonstrate that lauric acid activates TLR2 dimers as well as TLR4 for which respective bacterial agonists require acylated fatty acids, whereas DHA inhibits the activation of all TLRs tested. Thus, responsiveness of different cell types and tissues to saturated fatty acids would depend on the expression of TLR4 or TLR2 with either TLR1 or TLR6. These results also suggest that inflammatory responses induced by the activation of TLRs can be differentially modulated by types of dietary fatty acids.

Toll-like receptors (TLRs)[1] play a critical role in inducing innate immune responses by recognizing invading microbial pathogens (1–4). The activation of TLRs by agonists recruits an adaptor molecule, MyD88, and initiates the activation of downstream signaling cascades leading to the activation of NFκB and mitogen-activated protein kinase and the expression of inflammatory gene products, including cyclooxygenase-2 (COX-2), cytokines, and chemokines (2). Currently, eleven TLRs in mammalian cells are identified, and each TLR responds to different types of agonists: viral double-stranded RNA for TLR3, flagellin and single-stranded RNA for TLR5 and TLR7, respectively, and unmethylated *Cp*G DNA for TLR9 (3–5, 53–55). TLR4 recognizes lipopolysaccharide (LPS) derived from Gram-negative bacteria (6–8). TLR4 can be also activated by non-bacterial agonists such as heat shock protein 60, fibronectin, taxol, respiratory syncytial virus coat protein, and saturated fatty acids (9–13). TLR2 detects a variety of microbial components such as bacterial lipopeptides, peptidoglycan, and lipoteichoic acids (4). TLR2 forms a heterodimer with TLR1 or TLR6 to respond to and discriminate different types of agonists (4, 14). The activation of diacylated mycoplasmal lipopeptides, macrophage-activating lipopeptide 2-kDa (MALP-2), requires TLR2 dimerized with TLR6 to induce cytokine production, whereas the activation of TLR2 by triacylated bacterial lipopeptides requires the dimerization with TLR1 (15, 16). The saturated fatty acid moieties acylated in LPS and lipopeptides are critical for ligand recognition and receptor activation for TLR4 and TLR2. Deacylated LPS loses its endotoxic activity (17, 18), and the deacylated bacterial lipoproteins are unable to activate TLR2 and to induce cytokine expression in monocytes (19). Furthermore, LPS-containing unsaturated fatty acids is also inactive and acts as an antagonist against native LPS (20, 21).

Broadly, TLR agonists can activate two different downstream signaling pathways that are MyD88-dependent and -independent, leading to differential target gene expression and cellular responses. TLR2 and TLR9 induce NFκB activation and cytokine production through MyD88-dependent sig-

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[1] The abbreviations used are: TLR, Toll-like receptor; COX, cyclooxygenase; LPS, lipopolysaccharide; DHA, docosahexaenoic acid; NFκB, nuclear factor κB; MyD88, myeloid differentiation factor 88; IRAK-1, interleukin-1 receptor-associated kinase-1; TRAF6, tumor necrosis factor receptor-associated factor 6; IL-1, interleukin-1; TRIF, TIR domain-containing adapter inducing IFN-β; TICAM, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule; IFN-β, interferon β; IKK, IκB kinase; IRF3, IFN-regulatory factor 3; ISRE, interferon-stimulated regulatory element; PamCAG, palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)propyl)-Ala-Gly-OH: MALP-2, Macrophage-activating lipopeptide, 2 kDa; HA, hemagglutinin; FBS, fetal bovine serum; PGE_2, prostaglandin E_2; RT, reverse transcription; DN, dominant-negative; RLA, relative luciferase activity; CMV, cytomegalovirus.

naling pathways (22–24). The stimulation of TLR4 triggers the activation of both MyD88-dependent and -independent signaling pathways. The activation of TLR3 is known to stimulate primarily MyD88-independent signaling pathway. Toll-interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF)/TIR-domain-containing adaptor molecule (TICAM)-1 is an adaptor molecule responsible for activation of MyD88-independent pathways leading to the activation of IRF3 and the expression of IFNβ (25–27). Recently, it was reported that a new adaptor, TRIF-related adaptor molecule, interacts specifically with TRIF in an MyD88-independent pathway derived from the activation of TLR4, but not TLR3 (28, 29).

Results from our previous studies demonstrated that saturated and unsaturated fatty acids reciprocally modulate MyD88-dependent signaling pathways and target gene expression, including COX-2 derived from TLR4 activation (12, 30, 31). However, it is not known whether fatty acids also modulated MyD88-independent signaling pathways. The results from our previous studies showed that unsaturated fatty acids also suppressed NFκB activation and COX-2 expression induced by TLR2 agonist, a synthetic lipopeptide (PamCAG), in macrophages (30). In addition, saturated fatty acid potentiated TLR2 agonist-induced NFκB activation and COX-2 expression in macrophages (30). However, it remained to be determined whether the potentiation by the saturated fatty acid was mediated through the direct activation of TLR2. Moreover, it is not known whether fatty acids modulate the activation of other TLRs for which cognate ligands are not acylated by fatty acids.

TLR4 homodimerizes whereas TLR2 heterodimerizes with TLR6 or TLR1 (14–16, 32). Forced dimerization of TLR4 or TLR2 can lead to the activation of the downstream signaling pathways and target gene expression (1, 14, 32). These results suggest that the dimerization may be one of the initial steps for the activation of TLR2 or TLR4. Therefore, in this study, we investigated whether the activation of TLRs other than TLR4 and MyD88-independent signaling pathways are modulated by fatty acids and whether the dimerization of TLR4 or TLR2 is affected by different types of fatty acids.

EXPERIMENTAL PROCEDURES

Reagents—Sodium salts of saturated and unsaturated fatty acids were purchased from Nu-Chek (Eslyan, MN) and were dissolved in endotoxin-free water. Purified LPS was obtained from List Biological Laboratory Inc. A synthetic bacterial lipoprotein (PamCAG: palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)propyl)-Ala-Gly-OH) was purchased from Bachem (King of Prussia, PA). Poly(I:C) was purchased from Amersham Biosciences (Piscataway, NJ). Unmethylated *Cp*G DNA (ODN2006 and ODN1668) was purchased from TIB MolBiol (Berlin, Germany). Flagellin was obtained from Calbiochem (San Diego, CA). Macrophage-activating lipopeptide, 2 kDa (MALP-2) was purchased from Alexis Biochemical (San Diego, CA). Mouse monoclonal HA-antibody was obtained from Roche Applied Science (Indianapolis, IN). Mouse monoclonal FLAG-antibody was obtained from Sigma (St. Louis, MO). All other reagents were purchased from Sigma unless otherwise described.

Cell Culture—RAW264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T (human embryonic kidney cells; provided by Sam Lee, Beth Israel Hospital, Boston, MA) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). SW620 epithelial cells (ATCC CCL-227) were cultured in RPMI containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2/air environment. RAW264.7 cells stably transfected with murine COX-2 promoter (−3.2 kb) luciferase plasmid were prepared as described in our previous study (30).

Plasmids—pDisplay-FLAG-tagged TLR6 was prepared as follows: First, pDisplay-FLAG-tagged vector was obtained by the modification of pDisplay-HA-tagged vector (Invitrogen). A HindIII/BglII fragment in pDisplay-HA-tagged vector was replaced with a PCR product containing the coding sequence for FLAG peptide (KDDDDKYD), generated by the primers as follows: forward primer, 5′-CTATAGGGAGACCCAAGCTTGG-3′; reverse primer, 5′-GCGAGATCTCTTATCGTCGTCATCCTTGTAATCGTCACCAGTGGAACCTGGAAC-3′. The PCR product was digested with HindIII and BglII and ligated into pDisplay-HA-tagged vector generating pDisplay-FLAG-tagged vector. Next, PCR for mouse TLR6 was performed with the deletion of the endogenous leader sequence and the addition of BglII and XhoI sites at the two ends. The primers used for the PCR of TLR6 were as follows: forward primer, 5′-GCGAGATCTAATGAACTTGAGTCTATGGTAGAC-3′; reverse primer, 5′-GCGCTCGAGTCAAGTTTTCACATCATCCTCATTG-3′. The resulting product was ligated into BglII and SalI sites in pDisplay-FLAG-tagged vector. The integrity of the sequences was confirmed by DNA sequencing.

pcDNA-HA-TLR4 and pcDNA-FLAG-TLR4 were prepared as described in our previous studies (8). NFκB(2×)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). The luciferase reporter plasmid (pGL2) containing the promoter region of the murine COX-2 gene (−3.2 kb) was a kind gift from David Dewitt (Michigan State University, East Lansing, MI). The luciferase reporter plasmid containing the promoter of inducible nitric-oxide synthase was from Christopher Glass (University of California, San Diego). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). Mouse pDisplay-HA-TLR1, 2, 4, 6, and pDisplay-HA-TLR2(P>H) were obtained from Lynn Hajjar (University of Washington). Human pEF6-TLR5 was from Andrew Gewirtz (Emory University). Human pcDNA-TLR3 and a dominant-negative mutant of tumor necrosis factor receptor-associated kinase 6 (TRAF6) (pCMV4-TRAF6-(300–524)) were provided by Ruslan Medzhitov (Yale University School of Medicine). A dominant-negative mutant form of MyD88 (MyD88(ΔDD)) was kindly provided by Jurg Tschopp (University of Lausanne, Lausanne, Switzerland). A dominant-negative IL-1 receptor-associated kinase (IRAK)-1 (pCMV4-IRAK-1-(1–211)) was a kind gift from Sankar Ghosh (Yale University School of Medicine). A dominant-negative mutant of IKK (IKK(K44M)) was obtained from Michael Karin (University of California, San Diego). A dominant-negative mutant of inhibitor κB (pCMV4-IκBα(ΔN)) was provided by Dean Ballard (Vanderbilt University, Nashville, TN). A dominant-negative mutant of AKT (pSRα-AKT-T308A/S473A) was obtained from Bing-Hua Jiang (West Virginia University). A dominant-negative mutant of TRIF (TRIF ΔNΔC) was obtained from Shizuo Akira (Osaka University, Japan). A dominant-negative mutant of IRF3 (IRF3-DBD) was obtained from Genhong Cheng (University of California, Los Angeles, CA). All DNA constructs were prepared in large scale using the EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

Preparation of Bone Marrow-derived Macrophages and Measurement of Prostaglandin E_2 Production—Wild type (C3H/HeOUJ) and TLR4-mutant (C3H/HeJ) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Bone marrow cells isolated from femur were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 2 mM L-glutamine, 1 mM Na^+ pyruvate, 10 mM Hepes buffer, and 20% L929 cell-conditioned medium for 6 days, and adherent cells were used as macrophages. After treatment with lauric acid or docosahexaenoic acid in the absence or presence of LPS to bone marrow-derived macrophages, the levels of PGE_2 in the culture medium were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Real-time RT-PCR Analysis of COX-2 Expression in Mouse Bone Marrow-derived Macrophages—Bone marrow-derived macrophages from wild type (C3H/HeOUJ) and TLR4-mutant (C3H/HeJ) mice were treated with lauric acid, docosahexaenoic acid, or LPS for 4 h. Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Five micrograms of total RNAs were used for cDNA synthesis with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with a LightCycler (Roche Molecular Biochemicals) using the LightCycler FastStart DNA Master SYBR Green I kit. The primers used to detect mouse COX-2 are as follows: forward primer, 5′-ACACTCTATCACTGGCACCC-3′; reverse primer, 5′-GAAGGGACACCCCTTCACAT-3′ generating the amplified PCR product of 585 bp in length. The primers for mouse β-actin (used as an internal control) are as follows: forward primer, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; reverse primer, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′ generating the PCR product of 285 bp in length. The following program was used: denaturation at 95 °C for 10 min and 40 cycles consisting of denaturation at 95 °C for 1 s, annealing at 60 °C for 5 s, and extension at 72 °C for 25 s. The quality and specificity of the amplified PCR

products were assessed by performing a melting curve analysis and a conventional RT-PCR followed by agarose gel analysis. Samples were compared using the relative crossing-point value (*Cp*) method. The *Cp* value, which is inversely proportional to the initial template copy number, was determined by the LightCycler software program provided by the manufacturer (Roche Molecular Biochemicals). The -fold induction of COX-2 expression by real-time PCR was measured three times in duplicate relative to vehicle control and calculated after adjusting for β-actin using $2^{\Delta\Delta Cp}$, where $\Delta Cp = Cp_{\beta\text{-actin}} - Cp_{\text{COX-2}}$, and $\Delta\Delta Cp = \Delta Cp$ treatment $- \Delta Cp$ control.

Transient Transfection and Luciferase Assays—These were performed as described in our previous studies (8, 12, 30, 31). Briefly, RAW264.7 or 293T cells were co-transfected with a luciferase plasmid containing NFκB(2×)-binding site, murine COX-2 promoter (−3.2 kb), or inducible nitric-oxide synthase promoter, and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. SW620 cells were transfected with an IL-8 promoter-luciferase plasmid. Various expression plasmids or corresponding empty vector plasmids for signaling components were co-transfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector to eliminate the experimental error from transfection itself. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity to correct the transfection efficiency.

Immunoprecipitation and Immunoblot Analysis—These were performed essentially the same as previously described (8, 33, 34). Briefly, 293T cells were co-transfected with pDisplay-HA-TLR2 and pDisplay-FLAG-TLR6 (2 μg each). After 24 h, cells were washed with phosphate-buffered saline (pH 7.5) and lysed for 30 min on ice in lysis buffer (1% Nonidet P-40, 50 mM Hepes, pH 7.6, 250 mM NaCl, 10% glycerol, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium metabisulfite, 1 mM benzamidine hydrochloride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 12,000 × *g*, 4 °C for 15 min. Supernatants were incubated with 1 μg of HA antibody (12CA5) for 4 h and further incubated with 70 μl of 50% (v/v) protein A-agarose (Amersham Biosciences, Arlington Heights, IL) for overnight at 4 °C with rocking. Immune complexes were solubilized with Laemmli sample buffer after five times of washing with lysis buffer. The samples were fractionated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk and were blotted with the indicated antibodies. The reactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences). To reprobe with different antibodies, the membrane was stripped in the stripping buffer (35) at 56 °C for 1 h.

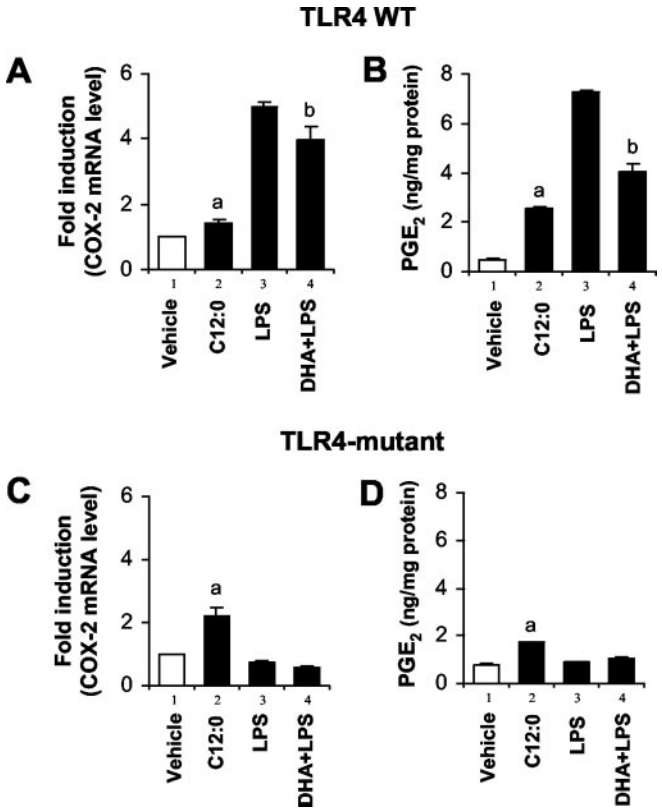

FIG. 1. **Lauric acid induces the expression of COX-2, but DHA inhibits LPS-induced COX-2 expression in bone marrow-derived macrophages isolated from wild-type and TLR4-mutant mice.** Bone marrow cells isolated from wild-type (C3H/HeOUJ) and TLR4-mutant (C3H/HeJ) mice were differentiated into macrophages and treated with lauric acid (C12:0, 100 μM) or DHA (C22:6*n*-3, 20 μM) in the presence or absence of LPS (100 ng/ml). *A* and *C*, after 4 h, total RNAs were extracted and the levels of COX-2 expression were determined by quantitative real-time RT-PCR analysis. COX-2 expression was normalized with β-actin (internal control) expression. The results are presented as -fold inductions compared with the vehicle control. *B* and *D*, after 18 h, the production of prostaglandin E_2 (*PGE_2*) in the culture medium was determined by enzyme-linked immunosorbent assay. Values are mean ± S.E. ($n = 3$). *a*, significantly different from *lane 1*, $p < 0.05$; *b*, significantly different from *lane 3*, $p < 0.05$. *WT*, wild-type.

RESULTS

Reciprocal Modulation of COX-2 Expression by Saturated and Polyunsaturated Fatty Acid in Bone Marrow-derived Macrophages Isolated from Wild-type and TLR4-mutant Mice—Results from our previous studies demonstrate that saturated fatty acid induces the activation of both endogenous and ectopically expressed TLR4 in macrophages (RAW264.7) and 293T cells, respectively, leading to NFκB activation and COX-2 expression (12, 30, 31). To determine whether the saturated fatty acid can activate other TLRs in addition to TLR4, macrophages derived from TLR4-mutant mice (C3H/HeJ), which express non-functional TLR4 but express other wild-type TLRs, were treated with the saturated fatty acid. Macrophages were prepared by differentiating bone marrow cells isolated from wild-type (C3H/HeOUJ) and TLR4-mutant (C3H/HeJ) mice. Cells were further treated with saturated fatty acid (lauric acid, C12:0) or polyunsaturated fatty acid (docosahexaenoic acid, DHA) in the presence or absence of LPS. Real-time RT-PCR analysis of COX-2 expression showed that lauric acid increased the steady-state level of COX-2 mRNA level, whereas DHA suppressed LPS-induced COX-2 mRNA in macrophages from wild-type mice (Fig. 1*A*). The levels of prostaglandin E_2 (PGE_2) produced during the incubation, which in part reflect the activity of COX-2 expressed, were increased by lauric acid, whereas DHA inhibited LPS-induced PGE_2 production in macrophages from wild-type mice (Fig. 1*B*). Consistent with results from our previous studies in RAW264.7 and 293T cells (12, 30, 31), COX-2 expression was also reciprocally modulated by saturated and polyunsaturated fatty acid in primary macrophages.

LPS-induced increase in COX-2 mRNA and PGE_2 level was completely abolished in macrophages from TLR4-mutant mice. In contrast, lauric acid increased the COX-2 mRNA level and PGE_2 production in TLR4-mutant macrophages (Fig. 1, *C* and *D*). These results suggest that saturated fatty acid may activate other TLRs in addition to TLR4 to induce COX-2 expression in macrophages.

Saturated Fatty Acid Does Not Activate TLR1, 2, 3, 5, 6, or 9 When an Individual TLR Is Ectopically Expressed in 293T Cells—To determine whether other TLRs are activated by saturated fatty acids, each TLR was ectopically expressed in 293T cells, and NFκB activation was determined after the treatment with lauric acid. Lauric acid did not induce NFκB activation when human TLR3, 5, or 9 was ectopically expressed in 293T cells, whereas the known agonist for each TLR induced NFκB activation (Fig. 2). The expression of individual TLRs 1, 2, or 6 in 293T cells was not sufficient to induce NFκB activation by

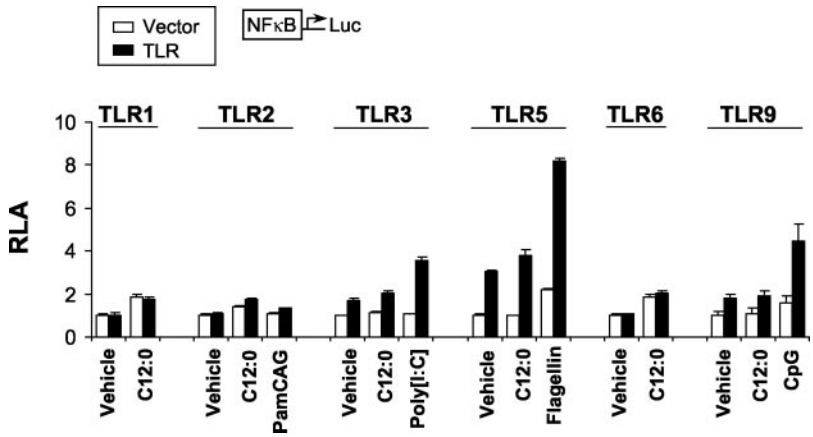

FIG. 2. **The ectopic expression of TLR1, 2, 3, 5, 6, or 9 alone in 293T cells does not confer the responsiveness to lauric acid.** 293T cells were co-transfected with NFκB(2x)-luciferase reporter plasmid and the expression plasmid of each TLR as indicated. As a vector control, pDisplay for mouse TLR1, 2, and 6, pcDNA for human TLR3, pEF6 for human TLR5, or pCMV for human TLR9, was transfected. After 24 h, cells were stimulated with PamCAG (20 ng/ml) for TLR2, poly(I:C) (10 μg/ml) for TLR3, flagellin (50 ng/ml) for TLR5, unmethylated CpG DNA (ODN2006, 10 μM) for TLR9, or lauric acid (C12:0, 75 μM) and further incubated for 18 h. Cell lysates were prepared, and luciferase and β-galactosidase enzyme activities were measured as described under "Experimental Procedures." Relative luciferase activity (*RLA*) was determined by normalization with β-galactosidase activity. Values are mean ± S.E. (n = 3). *PamCAG*, palmitoyl-Cys ((*RS*)-2,3-di(palmitoyloxy)propyl)-Ala-Gly-OH.

lauric acid or a TLR2 agonist, synthetic lipopeptide (PamCAG, 20 ng/ml) (Fig. 2). These results suggest that saturated fatty acid does not activate these TLRs when transfected alone and that saturated fatty acid is not a pan-agonist for the TLRs tested.

Saturated Fatty Acid Activates TLR2 Dimerized with TLR1 or TLR6—TLR2 requires dimerization with TLR1 or TLR6 to respond to certain agonists and induce the activation of downstream signaling pathways and cytokine expression (14, 32). Therefore, to determine whether TLR1 or TLR6 is required for the activation of TLR2 by saturated fatty acid, 293T cells were co-transfected with murine TLR2 and either TLR1 or TLR6. Lauric acid activated NFκB in a dose-dependent manner when TLR2 was co-transfected with TLR6 in 293T cells (Fig. 3, *A* and *B*). The activation of NFκB was also induced by lauric acid in 293T cells transfected with TLR2 and TLR1 (Fig. 3*A*). As previously shown in Fig. 2, low concentration of PamCAG (20 ng/ml) did not induce NFκB activation in 293T cells when TLR2 was transfected alone (Fig. 3*A*). However, PamCAG at higher concentration (1 μg/ml) induced NFκB activation in 293T cells transfected with TLR2 alone (data not shown). Another TLR2 agonist, MALP-2 (1 ng/ml) also induced NFκB activation in 293T cells transfected with TLR2 alone (Fig. 3*A*). These results suggest the minute expression of endogenous TLR1 and TLR6 in 293T cells. MALP-2 or PamCAG further potentiated NFκB activation when TLR2 was co-transfected with TLR1 or TLR6 (Fig. 3*A*). A dominant-negative (DN) mutant of TLR2 or TLR6, but not TLR4(DN), suppressed lauric acid-induced NFκB activation in 293T cells co-transfected with TLR2 and TLR6 (Fig. 3*C*). DHA inhibited NFκB activation induced by lauric acid in 293T cells transfected with TLR2 and TLR6 (Fig. 3*D*). Next, we determined whether lauric acid activates TLR2 dimers in another cell type. Lauric acid induced IL-8 promoter activity in human epithelial cells (SW620) transfected with human TLR2 and either TLR1 or TLR6 (Fig. 3*E*). Conspicuous induction of IL-8 induced by lauric acid in SW620 cells transfected with empty vector probably reflects the activation of endogenous TLR4. These results suggest that TLR2 requires the dimerization with TLR1 or TLR6 to confer the responsiveness to saturated fatty acid, MALP-2, and PamCAG.

A DN mutant of TLR1, TLR2, or TLR6 inhibited NFκB activation induced by lauric acid in macrophages (RAW264.7 cells) (Fig. 3*F*). These results demonstrate that the activation of NFκB by saturated fatty acid in macrophages is at least partly mediated through TLR2 dimerized with TLR1 or TLR6.

The Activation of TLR2 by Saturated Fatty Acid Leads to the Activation of MyD88-dependent Signaling Pathways—TLR2 is known to activate NFκB through MyD88-dependent signaling pathways involving IRAK-1, TRAF6, and IKK (22). To determine whether saturated fatty acid-induced TLR2 activation leads to the activation of these downstream signaling pathways, 293T cells were co-transfected with a DN mutant of each downstream signaling component along with TLR2 and TLR6. NFκB activation by lauric acid in 293T cells transfected with TLR2 and TLR6 was suppressed by the DN mutant of MyD88, IRAK1, TRAF6, IKKβ, or IκBα (Fig. 4, *A* and *B*). The activation of TLR2 can also lead to the activation of PI-3K/AKT resulting in enhanced transactivation of NFκB (36). In addition, results from our previous studies showed that AKT(DN) inhibited MyD88-induced NFκB activation. These results suggest that AKT is the downstream component of MyD88/PI-3K (31). Thus, we determined whether AKT is involved in the TLR2/6-signaling pathway activated by saturated fatty acid. AKT(DN) inhibited NFκB activation induced by lauric acid in 293T cells transfected with TLR2 and TLR6 (Fig. 4*C*). These results suggest that saturated fatty acid activates TLR2 leading to the activation of MyD88/IRAK1/TRAF6/IKKβ and MyD88/PI-3K/AKT pathways.

Saturated Fatty Acid Activates MyD88-independent Signaling Pathways through TLR4, but Not TLR2—In addition to the common MyD88-dependent signaling pathways, TLR4 can activate TRIF, a MyD88-independent adaptor molecule, leading to the activation of the transcription factor, IFN-regulatory factor 3 (IRF3), and the induction of IFN-β and IFN-regulated genes (28, 37–39). In contrast, TLR2 does not activate IRF3 or the IFN-β promoter (37, 38). Therefore, we determined whether saturated fatty acid can induce the activation of MyD88-independent signaling pathway using luciferase reporter gene driven by the interferon-stimulated regulatory element (ISRE), which has the binding site of IRF3 (28, 37). ISRE was activated by lauric acid and LPS in RAW264.7 cells (Fig. 5*A*). However, PamCAG, a TLR2 agonist, did not activate ISRE (Fig. 5*A*), although PamCAG induced NFκB activation in RAW264.7 cells as shown below (see Fig. 7*A*). The activation of ISRE induced by lauric acid was suppressed by TLR4(DN), but not by TLR2(DN) in RAW264.7 cells (Fig. 5*B*). In addition, this activation was inhibited by TRIF(DN) and IRF3(DN) (Fig. 5*B*).

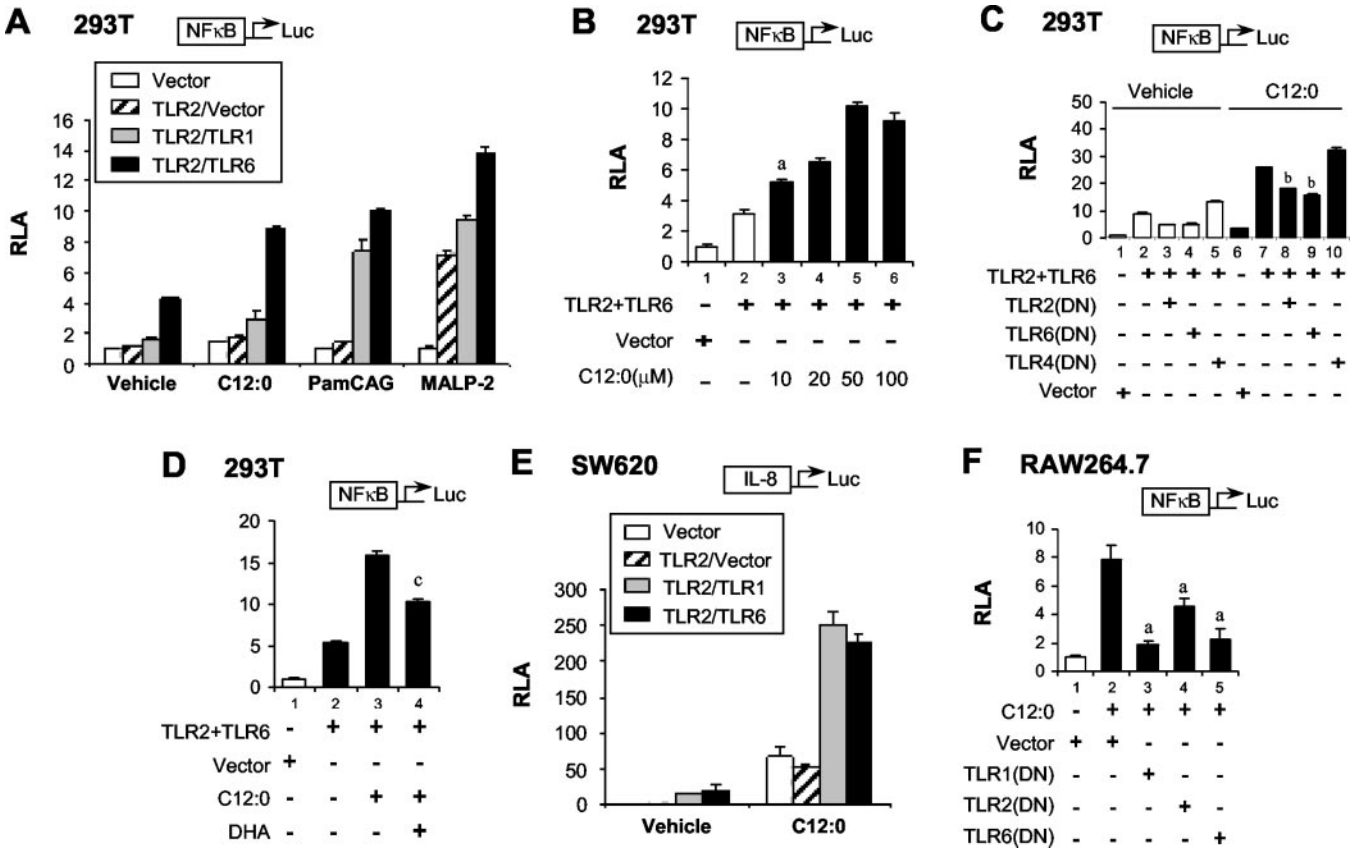

FIG. 3. **Lauric acid induces NFκB activation through TLR2 dimerized with TLR1 or -6.** 293T (*A–D*), SW620 (*E*), or RAW264.7 cells (*F*) were co-transfected with NFκB(2x)- (*A–D*, and *F*), or IL-8 promoter- (*E*) luciferase reporter plasmid, and the expression plasmids of wild-type or a dominant-negative (*DN*) mutant of TLRs as indicated. As a vector control, pDisplay for mouse TLR1, 2, 4, 6 (*A–D*, and *F*), or pCMV for human TLR1, 2, and 6 (*E*) was transfected. After 24 h, cells were stimulated with lauric acid (C12:0, 50 μM), PamCAG (20 ng/ml), MALP-2 (1 ng/ml), or DHA (20 μM) for 18 h (*A–D*, and *F*) or 8 h (*E*). Luciferase activities were determined as described in the legend for Fig. 2. Values are mean ± S.E. ($n = 3$). *RLA*, relative luciferase activity. *a*, significantly different from *lane 2*, $p < 0.05$. *b*, significantly different from *lane 7*, $p < 0.05$. *c*, significantly different from *lane 3*, $p < 0.05$.

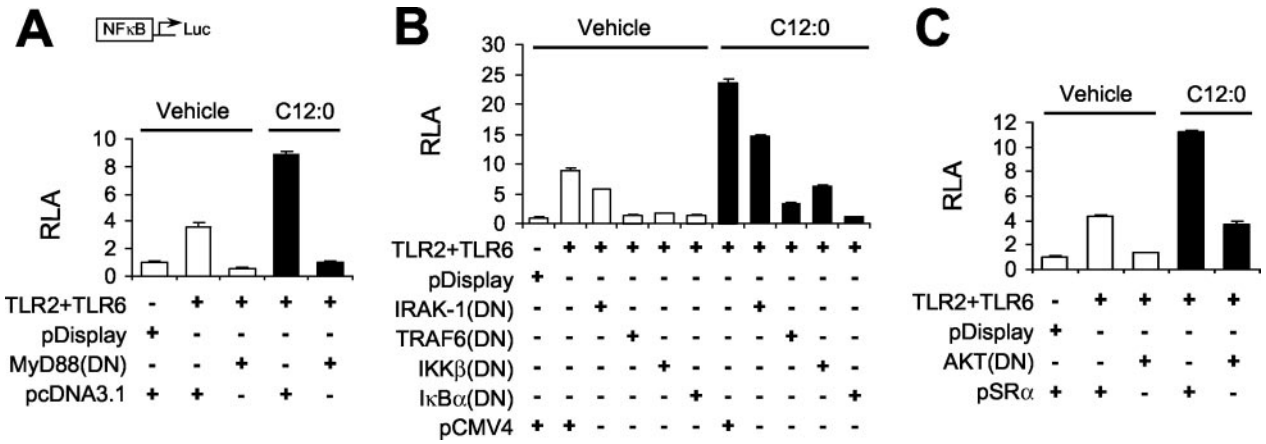

FIG. 4. **TLR2 activation induced by lauric acid leads to the activation of MyD88-dependent signaling pathways.** 293T cells were co-transfected with NFκB(2x)-luciferase reporter plasmid and various expression plasmids as indicated. As a vector control, pDisplay, pcDNA3.1, pCMV4, or pSRα was used as indicated in each panel. After 24 h, cells were stimulated with lauric acid (C12:0, 50 μM) for 18 h. Luciferase activities were determined as described in the legend for Fig. 2. Values are mean ± S.E. ($n = 3$). *RLA*, relative luciferase activity. *DN*, dominant-negative.

These results suggest that lauric acid activates the MyD88-independent signaling pathway mediated through the activation of TLR4 but not TLR2 in macrophages.

The Expression of COX-2 and iNOS Induced by Saturated Fatty Acid Is Mediated through the Activation of TLR2 in RAW264.7 Cells—We determined whether the activation of TLR2 by saturated fatty acid leads to the expression of target genes in macrophages. The expression of COX-2 and iNOS induced by lauric acid was suppressed by a DN mutant of TLR1, TLR2, or TLR6 in RAW264.7 cells (Fig. 6). These are consistent with the results demonstrating the inhibition of lauric acid-induced NFκB activation by a DN mutant of TLR1, TLR2, or TLR6 presented in Fig. 3 (*C* and *F*). These results suggest that saturated fatty acid-induced expression of inflam-

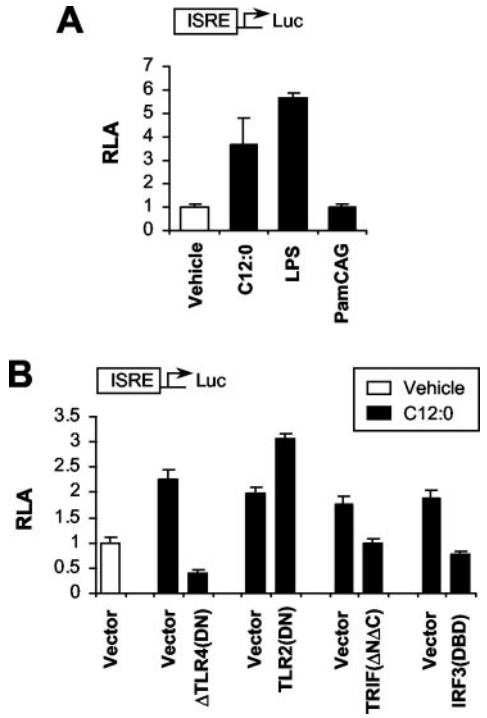

FIG. 5. **Lauric acid induces the activation of MyD88-independent signaling pathways mediated through the activation of TLR4, but not TLR2.** RAW264.7 cells were co-transfected with ISRE-luciferase reporter plasmid and various expression plasmids as indicated. As a vector control, pDisplay for TLR2(DN), pcDNA for ΔTLR4(DN), pCMV for TRIF(DN) (*TRIF(ΔNΔC)*), or pEBB for IRF3(DN) (*IRF3-DBD*) was used. After 24 h, cells were stimulated with lauric acid (C12:0, 75 μM), LPS (10 ng/ml), or PamCAG (1 μg/ml) for 18 h. Luciferase activities were determined as described in the legend for Fig. 2. Values are mean ± S.E. (n = 3). *RLA*, relative luciferase activity. *DN*, dominant-negative.

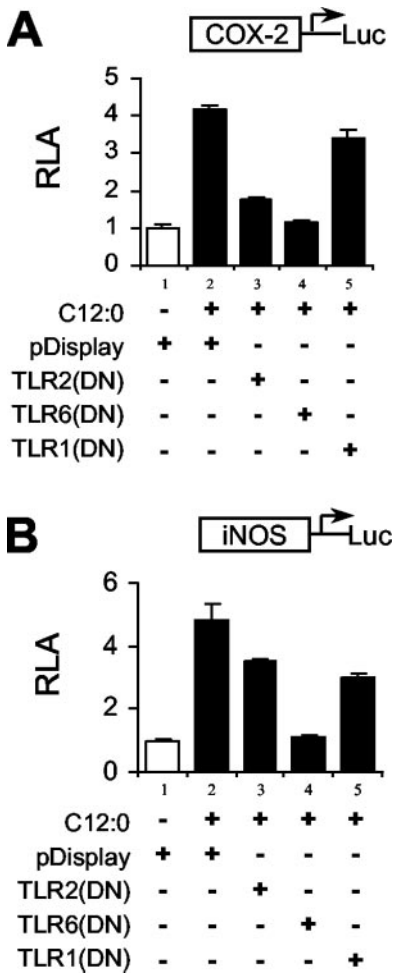

FIG. 6. **The expression of COX-2 and iNOS induced by lauric acid is inhibited by a dominant-negative (*DN*) mutant of TLR2, TLR1, or TLR6.** RAW264.7 cells were co-transfected with COX-2 promoter(−3.2 kb)-(*A*), or iNOS promoter-(*B*) luciferase reporter plasmid and the dominant-negative (*DN*) mutant of TLRs as indicated. Cells were further stimulated with lauric acid (C12:0, 75 μM) for 18 h. Luciferase activities were determined as described in the legend for Fig. 2. Values are mean ± S.E. (n = 3). *RLA*, relative luciferase activity.

matory gene products such as COX-2 and iNOS is at least partly mediated through the activation of TLR2 dimers in macrophages.

DHA Suppresses NFκB Activation and COX-2 Expression Induced by the Activation of TLR2, 3, 4, 5, or 9 in RAW264.7 Cells—Results from our previous studies showed that, contrary to saturated fatty acids, unsaturated fatty acids inhibited NFκB activation and COX-2 expression induced by LPS (TLR4 agonist) or lipopeptide (TLR2 agonist) in macrophages (30). The n-3-polyunsaturated fatty acid, DHA, was the most potent inhibitor among the unsaturated fatty acids tested. Therefore, we determined whether DHA inhibits the activation of other TLRs. DHA suppressed NFκB activation and COX-2 expression induced by various known TLR agonists, including PamCAG for TLR2, double-stranded RNA for TLR3, LPS for TLR4, flagellin for TLR5, or CpG for TLR9 in RAW264.7 cells as determined by reporter gene assays or immunoblotting analysis (Fig. 7). These results demonstrate that DHA inhibits the activation of TLR3, 4, 5, 9 as well as TLR2 dimerized with TLR1 or TLR6 in macrophages.

The Dimerization of TLR2 or TLR4 Is Not Affected by Saturated or Polyunsaturated Fatty Acid—Results from our previous studies showed that the molecular targets of saturated and unsaturated fatty acids are not the downstream signaling components of TLR4 (12, 31). Saturated and unsaturated fatty acids may affect the TLR itself or the proximal events leading to the activation of TLRs. The dimerization is considered to be a potential mechanism by which TLRs are activated, because forced dimerization of TLR4 or TLR2 leads to the activation of downstream signaling pathways (1, 14, 32). Therefore, we determined whether fatty acids modulate the heterodimerization of TLR2 and TLR6 using co-immunoprecipitation and immunoblotting of HA-tagged TLR2 and FLAG-tagged TLR6 ectopically expressed in 293T cells. The results show that TLR2 was co-immunoprecipitated with TLR6 demonstrating the heterodimerization of two receptors (Fig. 8, *A* and *B*). However, the heterodimerization was not affected by lauric acid or DHA (Fig. 8, *A* and *B*). We also determined whether the homodimerization of TLR4 is modulated by the fatty acids using ectopically expressed HA-tagged and FLAG-tagged TLR4 in 293T cells. Neither the fatty acids nor LPS (TLR4 agonist) were able to modulate the homodimerization of TLR4 (Fig. 8*C*).

DISCUSSION

Our results demonstrate that saturated fatty acid activates TLR2 dimerized with TLR1 or TLR6, but not TLR2 alone, whereas polyunsaturated fatty acid (docosahexaenoic acid, DHA) inhibits the activation of all TLRs tested. Saturated fatty acid activates MyD88-independent signaling pathways derived from the activation of TLR4, but not TLR2. Neither saturated fatty acid nor polyunsaturated fatty acid affected the dimerization of ectopically overexpressed TLR4 or TLR2 in 293T cells.

The results from our previous studies demonstrated that saturated fatty acids can activate TLR4 in macrophages (12). If the saturated fatty acid activates only TLR4 but not other TLRs,

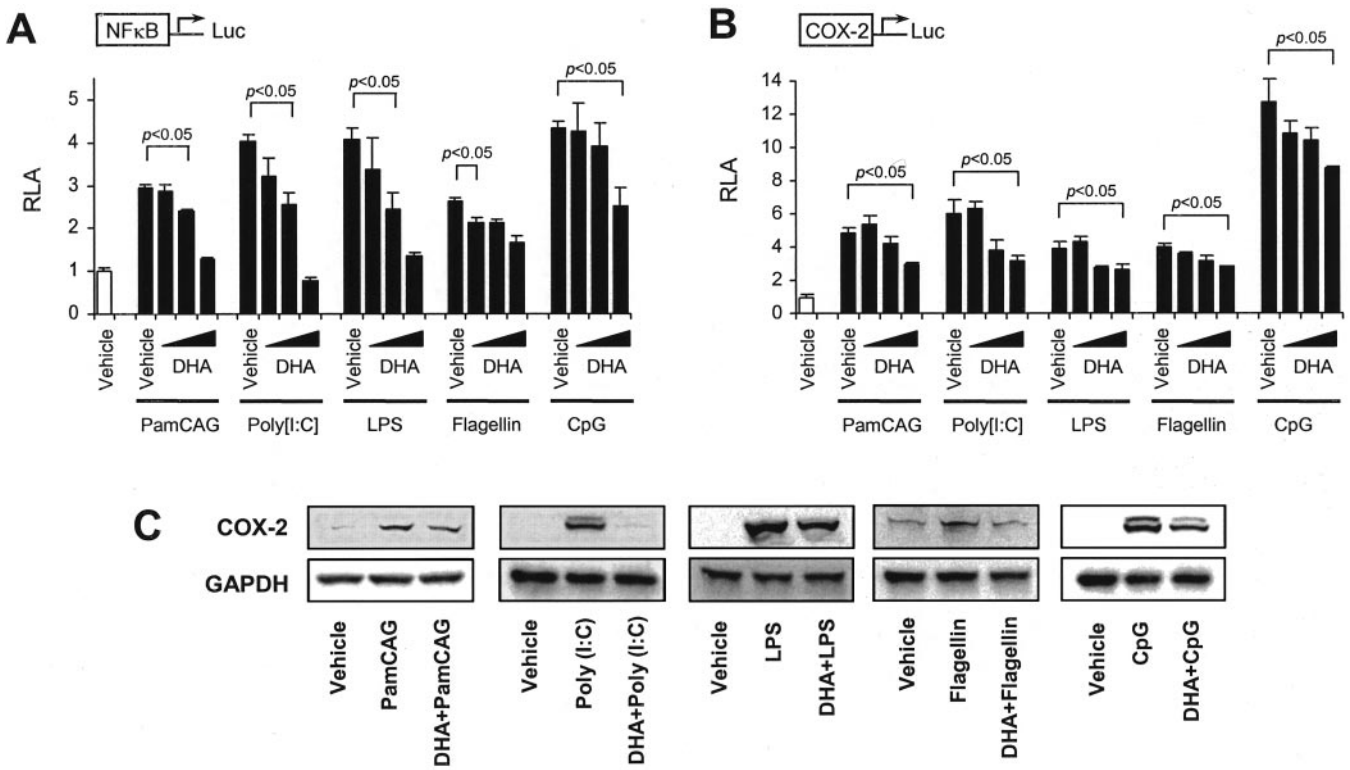

FIG. 7. **DHA suppresses NFκB activation and COX-2 expression induced by various TLR agonists.** RAW264.7 cells were transiently transfected with NFκB(2x)-luciferase reporter plasmid (*A*) or stably transfected with murine COX-2 promoter (−3.2 kb)-luciferase plasmid (*B*). Cells were pretreated with DHA (1, 2, and 5 μM for *A*; 1, 5, and 10 μM for *B*; 20 μM for *C*) for 1 h and further stimulated with PamCAG (1 μg/ml), poly(I:C) (10 μg/ml), LPS (100 ng/ml), flagellin (1 μg/ml), or unmethylated CpG DNA (ODN1668, 1 μM) for 8 h. Luciferase activities were determined as described in the legend for Fig. 2. Values are mean ± S.E. (n = 3). *C*, cell lysates were analyzed by COX-2 and GAPDH immunoblotting. The panels are representative data from more than two independent experiments.

macrophages derived from TLR4-mutant mice (C3H/HeJ), which express non-functional TLR4, would not be responsive to the saturated fatty acid. However, the results showed that the saturated fatty acid, but not LPS, induced COX-2 expression in TLR4-mutant macrophages. These results suggest that the saturated fatty acid can activate TLRs other than TLR4. Together with the results from our previous studies showing that saturated fatty acid activates both endogenously and ectopically expressed TLR4 (12, 31), the results demonstrate that saturated fatty acid activates TLR2 dimers and TLR4 for which cognate ligands from bacterial origin require acylated fatty acids in their molecules. However, saturated fatty acid failed to activate other TLRs for which the agonists do not appear to require acylated fatty acids for the receptor activation. These results suggest that responsiveness of different cell types and tissues to saturated fatty acids would require expression of TLR4 or TLR2 with either TLR6 or TLR1. Furthermore, our results suggest that TLR2, for which most known agonists are the microbial components, can be activated by non-microbial agonists, including saturated fatty acids. In contrast to the saturated fatty acid, n-3 polyunsaturated fatty acid (DHA) suppressed NFκB activation and COX-2 expression induced by various TLR agonists, *i.e.* TLR2, 3, 4, 5, and 9. The information on the responsiveness of different TLRs to types of fatty acids is critically important to evaluate the sensitivity of particular cell types and tissues with different TLR expression profiles to dietary fatty acids. The information will also help us understand how different types of dietary fatty acids can modify the risk of development of many chronic inflammatory diseases.

The two major downstream signaling pathways of TLRs are MyD88-dependent and -independent pathways, which are differentially activated by each TLR agonist leading to differential target gene expression and cellular responses. MyD88 is considered as a common downstream adaptor molecule for various TLRs to induce the expression of inflammatory cytokines. Synthetic bacterial lipopeptides (TLR2 agonist), imidazoquinoline (TLR7 agonist), and CpG DNA (TLR9 agonist) do not induce the activation of NFκB or the expression of cytokines in MyD88-deficient macrophages (22–24, 40). However, LPS (TLR4 agonist) can still activate NFκB in a delayed fashion and induce the maturation of dendritic cells in MyD88-deficient cells (24, 41–43). It has been shown that TRIF/TICAM-1 mediates the MyD88-independent signaling pathway of TLR4, leading to the activation of IRF3 and the expression of IFNβ and IFN-inducible genes (25–27). Our results demonstrate that TLR2 activation by the saturated fatty acid leads to the activation of MyD88-dependent signaling pathways (Fig. 4). The results also showed that saturated fatty acid activated a MyD88-independent promoter reporter gene mediated through TLR4/TRIF/IRF3, but not TLR2 (Fig. 5). These results suggest that the saturated fatty acid activates both MyD88-dependent and -independent signaling pathways.

TLR2 requires heterodimerization with TLR1 or TLR6 to confer the responsiveness to agonists (14–16, 32). It is also known that the forced dimerization of TLR4 using a chimeric TLR4 fused with CD4 or extracellular domain of integrin endowed a constitutive activity of TLR4 to activate the downstream signaling pathways leading to NFκB activation (1, 14, 32). These results suggest that dimerization may be one of the important initial steps for TLR2 and TLR4 to activate the downstream signaling pathways and target gene expression. The fact that saturated fatty acid activates TLR4 and TLR2, which dimerize with the corresponding partner TLR, suggests that the modulation of the dimerization step by saturated fatty acid may be a mechanism by which TLR2 and TLR4 are activated. However, the co-immunoprecipitation results showed that the saturated and polyunsaturated fatty acids did not

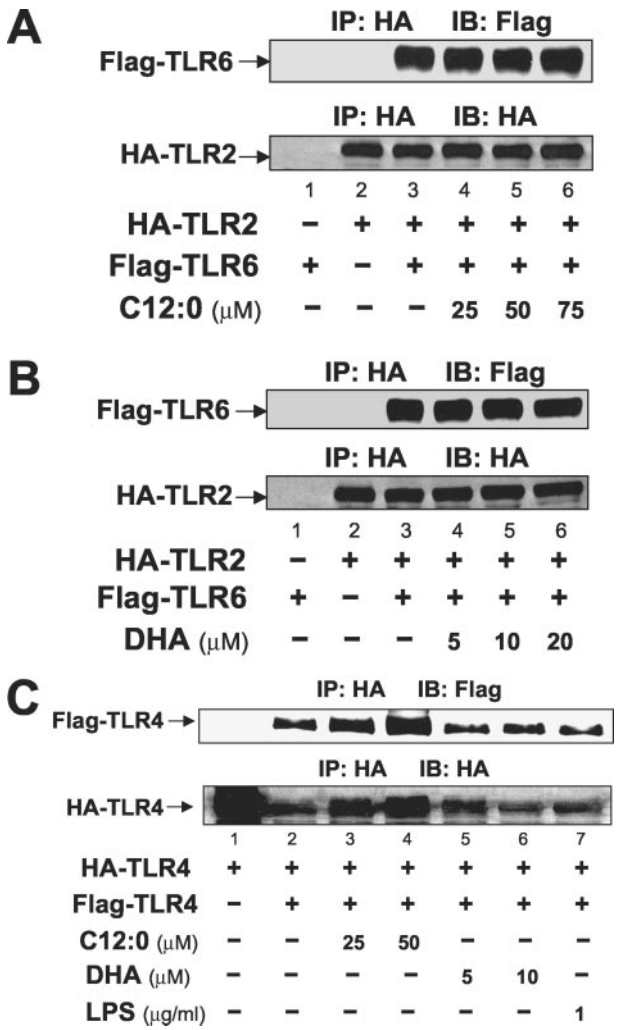

FIG. 8. **Lauric acid and DHA do not modulate the heterodimerization of TLR2 and TLR6 or the homodimerization of TLR4.** 293T cells were co-transfected with (*A* and *B*) pDisplay-HA-TLR2 and pDisplay-FLAG-TLR6 or (*C*) pcDNA-HA-TLR4 and pcDNA-FLAG-TLR4. After 24 h, cells were treated with lauric acid (C12:0), DHA, or LPS for 18 h. Cell lysates were immunoprecipitated with HA-antibody. Co-immunoprecipitated proteins were immunoblotted with FLAG-antibody and reprobed with HA antibody. The panels are representative data from three independent experiments.

affect the heterodimerization of TLR2 with TLR6 or the homodimerization of TLR4 when TLRs were ectopically expressed in 293T cells. LPS (TLR4 agonist) also did not modify the dimerization of TLR4 in 293T cells. Ozinsky *et al.* (14) also showed that peptidoglycan, TLR2 agonist, did not alter heterodimerization of TLR2 and TLR6 as determined by co-immunoprecipitation assays. Because co-immunoprecipitations were performed with overexpressed TLRs and overexpression itself induced the heterodimerization without agonist, we can not rule out the possibility that, in physiological states, agonists may induce the heterodimerization of endogenous TLRs to initiate the downstream signaling cascade leading to target gene expression.

The notion that DHA inhibits various TLRs implies that the molecular target of unsaturated fatty acid to inhibit TLR activation may be common to all TLRs. The potential target of unsaturated fatty acid may be the TLR itself or the proximal events leading to TLR activation but not the downstream signaling components, because DHA does not inhibit MyD88-induced NFκB activation and COX-2 expression (12, 30). It was demonstrated that LPS induces the co-clustering of CD14 and TLR4 and the recruitment of MyD88 in the lipid rafts (44, 45). Many signaling adaptor molecules and receptors are concentrated in lipid rafts upon agonist stimulation. Recently, Walton *et al.* (46) reported that oxidized phospholipid products, which suppressed LPS-induced IL-8 production, inhibited the LPS-induced translocation of TLR4 and MD2 to lipid raft/caveolae. It was reported that *n*-3 polyunsaturated fatty acid caused the alteration of fatty acid composition in membrane lipid rafts resulting in the inhibition of T-cell activation (47, 48). Whether unsaturated fatty acids modulate TLR activation mediated through the translocation of TLR and downstream signaling components to lipid rafts needs to be determined in future studies.

Polyunsaturated fatty acids can be enzymatically or non-enzymatically oxidized. Whether or not DHA itself or its oxidation products mediate the inhibitory effect on TLR activation remains to be determined. It has been reported that oxidized products of phospholipids have anti-inflammatory activities, whereas unoxidized phospholipids do not show the effect (49). Oxidized phospholipid products inhibited neutrophil binding, E-selectin expression, and NFκB activation induced by LPS in endothelial cells (49, 50). Bochkov *et al.* (50) suggested that oxidized phospholipids interfere with binding of LPS to LPS-binding protein and CD14, leading to the impairment of LPS binding to the TLR4 complex. In contrast, in other studies, oxidation products of phospholipids stimulated endothelial cells to produce inflammatory gene products such as MCP-1 and IL-8 and increased monocyte binding to endothelial cells (49, 51) suggesting the pro-inflammatory role of oxidized phospholipids. Nevertheless, a report that phosphatidylglycerol containing an unsaturated fatty acid, but not a saturated fatty acid moiety, inhibited LPS-induced NFκB activation (52) suggests that the inhibitory effect of phosphatidylglycerol depends on the types of acylated fatty acid, but not on the phospholipid backbone.

TLRs were evolved to detect invading pathogens and to induce innate immune responses to mount host defense mechanisms. It becomes apparent that the activation of some TLRs is also modulated by endogenous molecules, including fatty acids. Results from epidemiological and animal studies demonstrated that saturated and polyunsaturated dietary fatty acids can differentially modify the risk of development of many chronic diseases. Inflammation is now recognized as an important underlying etiologic condition for the pathogenesis of many chronic diseases. Therefore, if the activation of TLRs and consequent inflammatory and immune responses are also differentially modulated by types of fatty acids *in vivo*, this would suggest that the risk of chronic inflammatory diseases and host defense against microbial infection may also be modified by dietary fatty acids.

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